Specific Binding of Human Immunodeficiency Virus Type 1 (HIV-1) Gag-Derived Proteins to a 5′ HIV-1 Genomic RNA Sequence

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We developed an in vitro binding assay to study the specific interaction between human immunodeficiency virus type 1 (HIV-1) RNA and the Gag polyprotein. Binding of the in vitro-expressed protein to in vitro-transcribed RNA was determined by altered migration of the protein in polyacrylamide gels. We found that a Gag precursor lacking the matrix domain bound specifically to HIV-1 RNA, while deletion of both matrix and capsid domains diminished the specificity of binding. Among several regions of HIV-1 RNA tested, strongest binding was seen with the 5′-most 261 nucleotides, while antisense RNA from the same region did not bind.

The precise packaging of the viral genomic RNA into retroviral particles requires specific interactions between a *cis*-acting sequence in the viral RNA (called Ψ, or E) and the viral Gag polyprotein (reviewed in reference 20). For Rous sarcoma virus, murine leukemia virus, and spleen necrosis virus (13, 28–30, 32), Ψ sequences have been located at the 5′ end of the genome. For human immunodeficiency virus type 1 (HIV-1), sequences between the splice donor and start of the HIV-1 *gag* gene have been implicated in packaging (2, 6, 18). Deletion of these sequences, however, decreases genome packaging by only about fivefold (6, 16a, 22). Recently, another region of the HIV-1 genome at the 5′ end of the coding region for the Gag polyprotein has been shown to be important for Gag recognition and packaging (5, 21, 22). Thus, for HIV-1, there may be several regions contributing to specific genomic encapsidation.

The retroviral Gag protein is synthesized as a polyprotein which is cleaved by a virally encoded protease shortly after, or concomitant with, viral budding (12, 25). In HIV-1, cleavage results in four major mature proteins, matrix (MA), capsid (CA), nucleocapsid (NC), and p6. NC, which has been shown to specifically bind to several regions of the HIV-1 RNA (4, 5, 7, 21), contains two cysteine-histidine (Cys-His) boxes separated by a region of basic amino acids. Deletion of one Cys-His box allows RNA binding and packaging, but deletion of both boxes abrogates both functions (2, 4, 5, 7, 10, 23). The basic region is also required for binding in vitro (7, 31). However, it is not clear that cleaved mature NC is present at the time that RNA is selected for encapsidation. It is therefore more likely that the uncleaved Gag polyprotein is involved in RNA selection and that the NC Cys-His boxes and basic amino acids within the context of the uncleaved Gag precursor are involved in specific association with genomic RNA.

In this study, we have looked at the in vitro binding of various truncated forms of the HIV-1 Gag polyprotein to RNAs derived from different regions of the HIV-1 genome.

We used an in vitro binding assay, in which in vitro-tran-

scribed ³H-labeled RNA was combined with in vitro-synthesized, ³⁵S-labeled, deleted forms of the Gag protein. RNA-protein complexes were detected on a nondenaturing polyacrylamide gel, on which their migration is different from that of free protein. This type of gel-shift assay differs from more conventional ones in that the labeled protein is detected rather than the nucleic acid. An assay of this type had previously been used to study binding of Sindbis virus capsid protein to RNA (9).

For in vitro transcription of HIV-1-derived RNA, various regions of the HIV-1 genomic cDNA (pBru3 [26]; GenBank accession no. K02013) were cloned in one or both orientations under the control of a viral T7 DNA-dependent RNA polymerase promoter in the pSP73 vector (Promega, Madison, Wis.) such that after linearization of the DNA with appropriate restriction endonucleases the sense or antisense strand could be transcribed (Fig. 1A). Nonspecific RNAs were transcribed from control plasmids as described in Table 1. In vitro transcription from the SP6 or T3 DNA-dependent RNA polymerase promoter was performed under standard conditions (15). For transcriptions with T7 DNA-dependent RNA polymerase, RNA yields were maximized by using the conditions described previously (24). Transcripts were labeled by incorporation of ³H-UTP and the yields were quantified by trichloroacetic acid precipitation. All transcripts were routinely analyzed for size and intactness on denaturing 7 M urea–6% polyacrylamide gels.

HIV-1 Gag deletion proteins were expressed in a rabbit reticulocyte lysate-based coupled in vitro transcription-translation system (Promega), following the manufacturer's protocol. Uncut plasmid DNA, containing the HIV-1 coding sequence under the control of an SP6 promoter and upstream of a $(dA)_{30}$ stretch, was used as the template. The details of plasmid construction are given in Fig. 1B. Initiation of translation depended on the selection of the first AUG on the mRNA as the initiation codon, which in all cases was in frame with the Pr55^{Gag} open reading frame. For the N-terminal deletion mutants of Gag, the AUG utilized is an internal methionine codon in the wild-type provirus context. Protein products were labeled by incorporation of [³⁵S]methionine. Incorporation was determined by trichloroacetic acid precipitation and typically ranged between 5 and 25% of the input, depending on the actual Gag fragment expressed. When analyzed on a 15%

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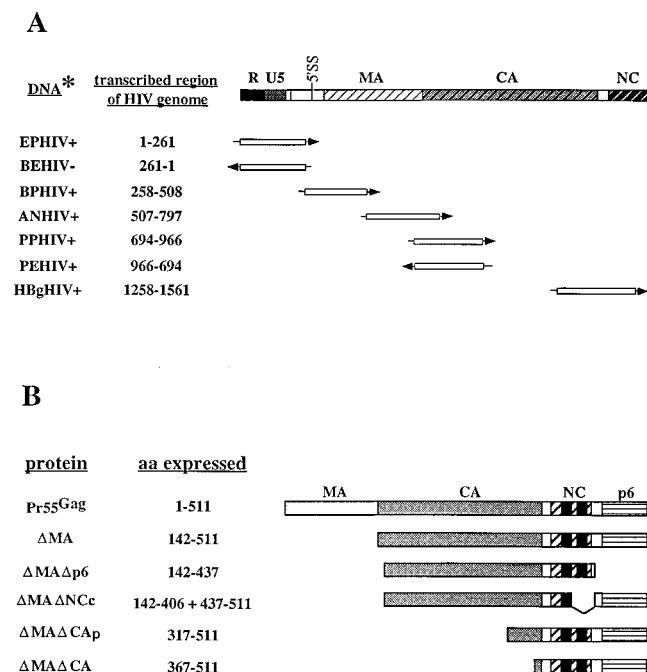
A

B

FIG. 1. In vitro-synthesized HIV-1-derived RNAs and Gag protein fragments. (A) Location of in vitro-transcribed RNAs on HIV-1 genome. Open boxes shown below the diagram of the 5′ end of the genome represent HIV-1-derived sequences, thin lines represent vector-derived stretches, and the arrowheads indicate the orientation [sense (+) or antisense (−)] of RNA. *, before transcription, DNAs were linearized at the following restriction enzyme sites: *Bss*HII (EPHIV), *Eco*RI (BEHIV), *Acc*I (BPHIV, ANHIV, and PPHIV), *Alw*NI (PEHIV), and *Avr*II (HBgHIV). (B) Diagram of Gag deletion proteins expressed in vitro. The Gag domains expressed are shown as boxes: NC is indicated by the diagonally striped boxes, and the position of the Cys-His boxes within the NC domain is highlighted by solid black areas. pPA6gag was constructed by inserting the entire Pr55^{Gag}-coding region followed by the first 178 nucleotides of the overlapping protease-coding region and preceded by 6 nucleotides of the 5′ untranslated sequence into the pSP64poly(A) vector (Promega). The 178 nucleotides derived from the protease-coding region are not sufficient to allow expression of an active protease (14). For construction of pΔMA, pΔMAΔCAp, and pΔMAΔCA, HIV-1-derived sequences upstream of the *Pvu*II site (position 694 in the HIV-1 sequence), the *Hin*dIII site (position 1259), and the *Ppu*MI site (position 1399), respectively, were deleted. Deletion of the HIV-1-derived sequences downstream of the *Bgl*II site (position 1642 in the HIV-1 sequence) and insertion of a 14-bp *Xba*I linker containing TAG stop codons in all three reading frames gave rise to pΔMAΔp6. pΔMAΔNCc was constructed by introducing an in-frame deletion from the blunt-ended *Apa*I to the filled-in *Bgl*II site (position 1553 to 1642 in the HIV-1 sequence). All deletion junctions were verified by dideoxy nucleotide sequencing by using the Sequenase version 2.0 kit from U.S. Biochemical Corp. (Cleveland, Ohio). aa, amino acids.

polyacrylamide–sodium dodecyl sulfate (SDS) gel (16), all translation products showed multiple bands (Fig. 2). The largest major band in each case migrates at a position consistent with the molecular mass of the expressed protein. ΔMA, ΔMAΔNCc, and ΔMAΔp6, all of which share the same N-terminal sequence, display an identical pattern of three bands, which is shifted according to the sizes of the proteins. The same pattern of three bands is also visible in the Pr55^{Gag} lane. Therefore, a likely explanation for the multiple bands is that they represent proteins initiated at successive AUG codons on the same mRNA.

For RNA-protein binding reactions, optimized amounts of in vitro-transcribed RNA (3 pmol) and the unpurified in vitro transcription-translation reaction mixture (1 to 2 μl) containing the specific HIV-1 protein were incubated in a volume of 10 μl. The actual amount of HIV-1-specific protein present in the binding reaction mixture is not known, but from the

TABLE 1. Summary of binding of ΔMA to in vitro-transcribed non-HIV-1-derived RNAs

RNA (length)[a]	Plasmid used[b]	Binding[c]
5′ 261 nt of HIV-1 (282)	pEPHIV (*Bss*HII)	+++
RSV Ψ (271)	pASY159 (*Hin*dIII)	+++
β-Globin promoter (276)	pSX (*Hga*I)	−
β-Galactosidase coding region (222)	pEC1206 (*Mlu*I)	−
Neomycin resistance coding region (318)	pBSneo (*Pvu*II)	−
pSP64 vector sequence (266)	pSP64poly(A) (*Pvu*II)	+
pSP73 vector sequence (186)	pSP73 (*Nde*I)	−

[a] Total length in nucleotides, including vector-derived sequences. RSV, Rous sarcoma virus.

[b] Restriction enzyme sites used to linearize plasmids for in vitro transcription are given in parentheses. pASY159 (provided by A. Yeo, Fred Hutchinson Cancer Research Center, Seattle, Wash.) contains nucleotides 357 to 628 of the Rous sarcoma virus sequence (GenBank accession no. J02342) inserted into pBSII (Stratagene, La Jolla, Calif.). pSX, which contains the *Sph*I-*Xba*I fragment of the sickle allele of the human β-globin gene inserted into pBS, was obtained from E. Epner and M. Groudine, Fred Hutchinson Cancer Research Center. pECL206, which carries the 339-bp *Taq*I fragment spanning the *Sst*I site of the *lacZ* gene inserted into pBS, was kindly provided by A. Geballe, Fred Hutchinson Cancer Research Center. pBSneo (provided by A. Yeo) has the neomycin gene inserted into pBSII.

[c] In vitro binding reactions and gel electrophoresis were carried out as described in the text. +++, strong binding; +, weak binding; −, no binding.

amount of trichloroacetic acid-precipitable [^{35}S]methionine, it is estimated to be at least 100-fold-lower than the amount of RNA. The binding reaction mixture contained 10 mM HEPES (*N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.2), 5 mM MgCl$_2$, 2 mM dithiothreitol, and 1 mg of heparin per ml. The addition of up to 200 mM KCl or 10 μM ZnCl$_2$ did not alter the detectable binding. After a 20-min incubation at room temperature, 1 μl of 50% glycerol was added, and the entire binding reaction mixture was loaded
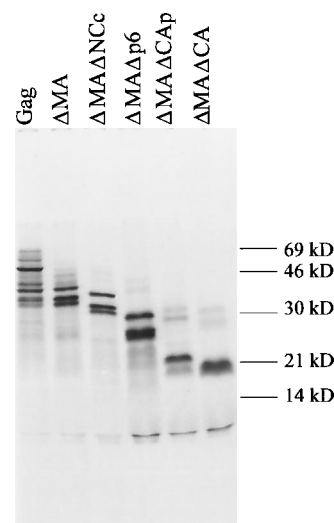

FIG. 2. SDS-PAGE analysis of in vitro translation products. A total of 2.5 μl of each 50 μl of the in vitro transcription-translation reaction mixture programmed with the plasmids indicated at the top of the lanes was loaded per lane; 2.5 μl of the nonradioactive, prestained molecular marker (Rainbow marker; Bethesda Research Laboratories, Bethesda, Md.) was run in parallel. After gel electrophoresis, the dried gel was lined up with the autoradiogram, and the positions of the colored marker protein bands were transferred to the autoradiogram. Sample preparation and gel electrophoresis were conducted according to the method of Laemmli (16).
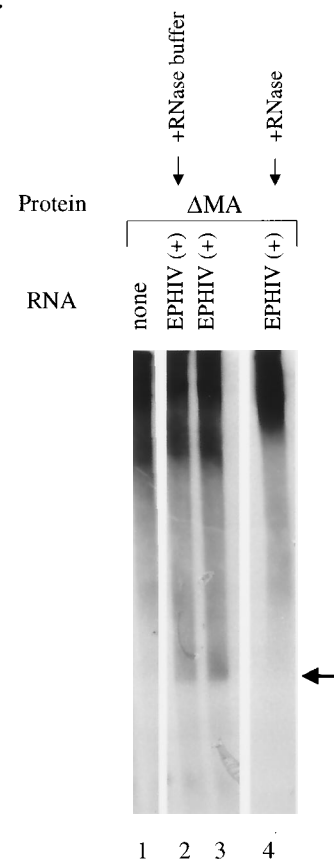
A.

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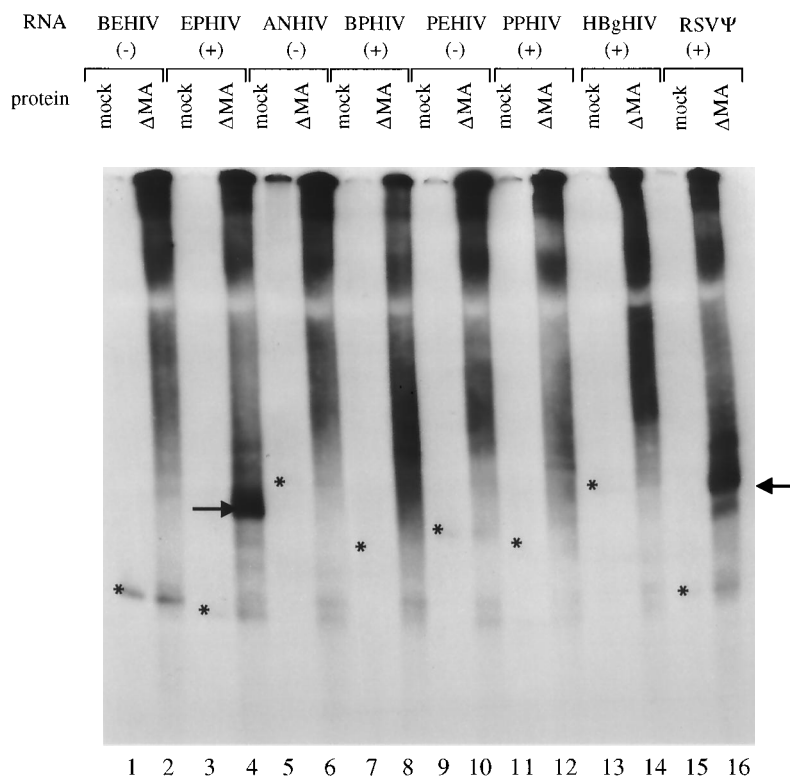
FIG. 3. In vitro ΔMA Gag protein-HIV-1 RNA binding assay. (A) Each transcription-translation reaction mixture (2 μl) containing no HIV-1 protein (mock; odd-numbered lanes) or ^{35}S-labeled ΔMA Gag protein (even-numbered lanes) was added to 3 pmol of ^{3}H-labeled RNA in binding buffer (see the text). The RNAs used, as indicated above the lanes, are described in the legend to Fig. 1. Lanes 15 and 16, ASY159 (HindIII) containing the Rous sarcoma virus Ψ site. Asterisks indicate the locations of the RNA band, in the lanes labeled mock, determined from a longer exposure of this autoradiograph. The arrows point to the major shifted protein-RNA complexes. All lanes illustrated are from the same gel. (B) A transcription-translation reaction mixture (2 μl) containing ^{35}S-labeled ΔMA protein was added to binding buffer (see the text) containing no HIV-1-specific RNA (lane 1) or 3 pmol of ^{3}H-labeled EPHIV+ RNA (lanes 2 to 4). After incubation for 20 min at room temperature, 1 μl of either 10 mM Tris (pH 7.6)–15 mM NaCl containing 1 μg of RNase A (lane 4) or RNase buffer alone (lane 2) was added, and the incubation continued for 30 min at room temperature before the gel was loaded. The arrow points to the major labeled complex. All lanes illustrated are from the same gel.

onto a 5% polyacrylamide–5% glycerol gel in 45 mM Tris-borate–1 mM EDTA (pH 8.5). The gel was run for 3 h at 170 to 200 V and fixed, and the radioactivity was visualized by fluorography. All binding experiments were repeated four or five times, and the results were reproducible.

Initial binding reactions were carried out with a ^{35}S-labeled matrix-domain-deleted form of the Gag polyprotein, ΔMA. It has been previously shown that the MA domain is not required for RNA encapsidation into virions (17). Several RNAs derived from different regions within the 5′ proximal part of the HIV-1 genomic RNA were tested. As controls, the antisense RNAs corresponding to two of these regions were also included. All RNAs are named according to the plasmid DNAs from which they were transcribed, and their orientation is indicated by + (sense) or − (antisense) (Fig. 1A) ^{3}H-labeled RNA and ^{35}S-labeled protein were combined in solution as described above, and binding was analyzed on a nondenaturing polyacrylamide gel (Fig. 3A). In the presence of a mock transcription-translation reaction mixture (no template DNA added; odd-numbered lanes), all RNAs migrate as single bands (the positions are indicated by asterisks; the ^{3}H-labeled RNA bands cannot be visualized at this exposure of the gel). On the

addition of a transcription-translation reaction mixture containing ΔMA, a smear appears along the top of the lanes. This diffuse signal originates from the ^{35}S-labeled protein, which does not run as a discretely sized band on a nondenaturing gel. The diffuse region of label may also represent binding to unspecific RNA or protein-protein complexes of various sizes. On the addition of ΔMA to EPHIV+ RNA (lane 4), a strong and discrete new band appears. In order to show that this band represents a complex between ΔMA and EPHIV+ RNA, RNase was added in a control experiment (Fig. 3B). While ΔMA by itself shows only a smear (Fig. 3B, lane 1), the addition of EPHIV+ RNA results in a new band (Fig. 3B, lanes 2 and 3), which disappears after treatment with RNase (Fig. 3B, lane 4). In contrast, no new bands are visible when ΔMA is added to BEHIV−, ANHIV+, PEHIV−, or PPHIV+ RNA (Fig. 3A, lanes 2, 6, 10, and 12, respectively). The addition of ΔMA to BPHIV+ and HBgHIV+ RNAs (lanes 8 and 14, respectively), and in some experiments to PPHIV+ and PEHIV− RNAs (data not shown), also results in new bands. These signals, however, are significantly weaker and less defined than the one seen with EPHIV+ RNA (lane 4). Thus, in our in vitro binding assay, an MA-domain-deleted form of

Pr55^{Gag}, ΔMA, shows the strongest binding to EPHIV+ RNA, which contains the 5′ 261 nucleotides of the HIV-1 genomic RNA. No binding to the corresponding negative-strand RNA, BEHIV−, or a number of RNAs from other regions of the HIV-1 genome is seen. However, in addition to the 5′ 261 nucleotides, several other regions of the HIV-1 RNA also bound to the Gag fragment, although not as consistently, and the complexes formed were more diffuse. One of these RNAs (BPHIV+) covers the region around the start codon of the Gag protein, which contains sequences previously shown to be important for binding to Gag protein and for packaging (2, 6, 18, 21, 22). A survey of the literature suggests that there may be more than one region of the HIV-1 genome which is involved in RNA packaging. There is not as yet any evidence for a single region of HIV-1 RNA which is sufficient to allow encapsidation of heterologous RNA, as is the case for oncoretroviruses (1, 3). It may be that several regions of the HIV-1 RNA must combine to form a complex Ψ structure or that several independent Ψ elements may have to be bound by one or more Gag proteins before assembly of the viral capsid and concomitant packaging of the genomic RNA are triggered.

To further explore the specificity of the in vitro binding of ΔMA to RNA, we tested a number of unrelated RNAs (summarized in Table 1). No binding to RNAs transcribed from the promoter region of the human β-globin gene, from the coding region of the β-galactosidase gene or the neomycin gene, or from the pSP73 plasmid used for cloning the HIV-1 RNAs was detected. However, there was strong binding to an RNA containing the packaging region of Rous sarcoma virus (3) (also shown in Fig. 3A, lane 16). Berkowitz et al. (5) also found binding of a bacterially expressed Gag protein to packaging regions from bovine leukemia virus and Mason-Pfizer monkey virus. These findings indicate that some general feature of the Ψ structure is conserved among retroviruses and that this feature is recognized in in vitro binding assays. We also detected weaker but significant binding to RNA transcribed from the pSP64poly(A) plasmid. It is not possible to say whether this RNA forms a structure similar to that of HIV-1 Ψ or whether this result reflects some lack of specificity in our assay.

It was difficult to examine the binding properties of the full-length Gag protein in our assay system since the addition of EPHIV+ RNA did not change the pattern of bands already seen with the protein alone (data not shown). It is likely that the full-length Gag protein binds to its own mRNA in the in vitro transcription-translation reaction, which at its 5′ end contains sequences previously implicated in specific binding to Pr55^{Gag} (21, 22).

In order to determine the domains of the ΔMA Gag protein which contribute to binding specificity, additional constructs encoding deleted forms of the protein were generated. These derivatives of HIV-1 Gag lack the MA and p6 proteins (ΔMAΔp6), MA and part of the CA proteins (ΔMAΔCAp), MA and the entire CA domains (ΔMAΔCA), and MA protein and the region of the NC protein encompassing the second Cys-His box and most of the basic region in between the two Cys-His boxes (ΔMAΔNCc; see Fig. 1B). ΔMA, ΔMAΔNCc, ΔMAΔp6, ΔMAΔCAp, and ΔMAΔCA were incubated in the presence of either BEHIV− or EPHIV+ RNA under standard binding conditions and analyzed on a nondenaturing gel (Fig. 4). ΔMAΔCAp and ΔMAΔCA do not show the background smear seen with the other Gag deletion proteins, possibly because in these two smallest proteins tested, the basic nature of the NC domain becomes more apparent and prevents migration of the proteins into the gel.

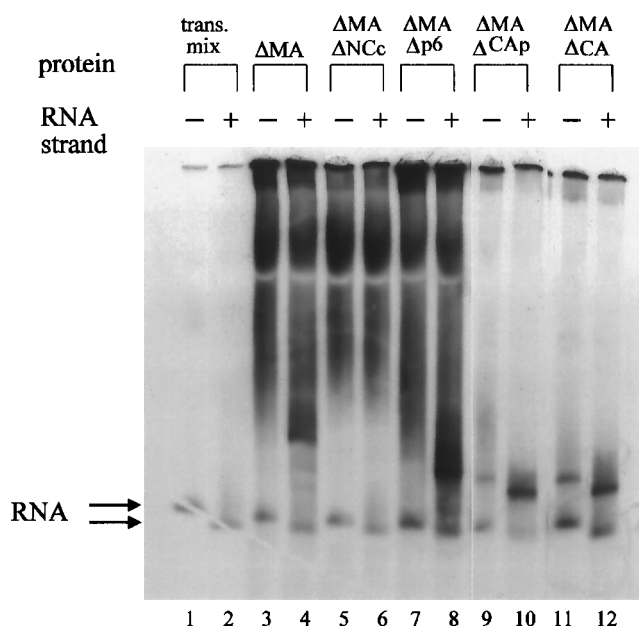In the presence of sense EPHIV+ RNA, the addition of ΔMA (Fig. 4, lane 4), ΔMAΔp6 (lane 8), ΔMAΔCAp (lane 10),

FIG. 4. In vitro binding of various Gag deletion proteins to 5′ end of HIV-1 RNA. BEHIV− (lane 1) or EPHIV+ RNA (lane 2) in binding buffer (see the text) was incubated with 2 μl of transcription-translation reaction mixture containing no HIV-1 protein (trans. mix). To test the binding specificity of the Gag deletion proteins, 2 μl of the translation mix containing ΔMA (lanes 3 and 4), ΔMAΔNCc (lanes 5 and 6), ΔMAΔp6 (lanes 7 and 8), or ΔMAΔCAp (lanes 9 and 10) or 1 μl of the translation mixture containing ΔMAΔCA together with 1 μl of the mock reaction mixture (lanes 11 and 12) was added to EPHIV+ RNA (even-numbered lanes) or BEHIV− RNA (odd-numbered lanes). The two arrows indicate the locations of the RNA only bands (top arrow shows BEHIV− and the bottom arrow EPHIV+). All lanes illustrated were from the same gel.

or ΔMAΔCA (lane 12) gives rise to a fairly discrete, shifted band. The size of the shift decreases from ΔMA to ΔMAΔp6 to ΔMAΔCAp and ΔMAΔCA, consistent with the decreasing size of the protein binding to the RNA. No binding is detected with ΔMAΔNCc (Fig. 4, lane 6) or with a deletion construct lacking the first Cys-His box as well as part of the internal basic region of the NC protein (data not shown). For both ΔMA and ΔMAΔp6, a new ^{35}S-containing band appears only on the addition of EPHIV+ RNA but not BEHIV− RNA. However, in the case of ΔMAΔCAp and ΔMAΔCA, a weaker but distinct band is also visible with BEHIV− RNA. ΔMAΔCAp and ΔMAΔCA thus display less-specific binding to HIV-1 RNA than do ΔMA and ΔMAΔp6, and the CA domain must therefore contribute to the specificity of RNA binding. Since no independent RNA binding could be demonstrated for the CA domain (see below), CA probably acts by increasing the specificity of binding of the NC domain. A contribution of the CA domain to the specificity of RNA binding would meet the requirements of virus assembly in vivo. During infection, the HIV-1 RNA must first be specifically selected from the pool of intracellular RNAs by the Gag precursor, while after protease cleavage in the virion, the entire viral RNA must be sequence independently coated by the NC protein.

Several studies regarding the specific RNA binding activity of the NC domain in vitro have been published (7, 19). In only one study, however, was RNA binding of the NC compared with that of a Gag protein also containing the CA domain (5). Using bacterially expressed glutathione S-transferase (GST) fusion proteins, these investigators found comparable specific RNA binding activities for the full-length Gag and the NC proteins. However, only the GST fusion proteins and not the

cleaved fragments were tested for their RNA binding activity. It is conceivable that the GST domain acts much like the CA domain in altering the conformation of the NC domain to confer specificity on RNA binding. It should also be noted that all of these investigators used different regions of the HIV-1 RNA as specific binding substrates.

A Gag fragment with deletions of the entire MA domain and either the first or the second Cys-His box as well as part of the basic region of the NC domain showed no RNA binding activity in our assay. This result confirms the importance of the Cys-His boxes as well as the basic region between those two boxes within NC, which has been well documented in the literature (2, 5, 8, 10, 11, 27). It also allows the conclusion that neither the CA nor the p6 domain, in the context of the partially deleted NC domain, has independent RNA binding activity strong enough to be detected in our assay system. With a different in vitro binding system, it was reported that there is residual specific RNA binding activity of a GST-Gag fusion protein deleted in either the first or the second Cys-His box of the NC domain (5). However, these investigators also reported a sharp, nonlinear drop in RNA-protein complex formation, when the protein concentration was lowered. In our assay, the Gag protein concentration is probably very low due to its expression in an in vitro translation system. Therefore, it is not surprising that in our assay, a Gag fragment carrying a deletion in part of the NC domain shows no RNA binding activity at all.

In summary, our in vitro binding assay confirms that the MA and p6 regions of Gag are not required for binding to RNA and that the Cys-His box and basic region of NC are essential for binding to RNA. In addition, we show that deletion of the CA domain decreases the specificity of binding. The results presented here suggest that the 5′ end of the HIV-1 genome contains information which is important for the association with the viral Gag protein and therefore may be involved in genomic encapsidation. This region could act in concert with other regions of the genome to ensure efficient encapsidation of genomic RNA.

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